



# Exploring the impact of formulated bacterial consortia on the growth of *Solanum lycopersicum*; towards sustainable biofertilizer development

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## Abstract

Biofertilizers are a sustainable solution for the adverse biogeochemical impacts exerted by synthetic agrochemicals. The application of biofertilizers is an eco-friendly approach to facilitate plant growth and development. Biofertilizers are mainly composed of living or dormant microbes, which are usually applied to the soil. These microbes encourage plant growth by a wide variety of mechanisms which includes phytohormone production, nutrient solubilization, biocontrol ability, etc. In the present study, bacterial cultures were isolated from nutrient-rich sample sources and screened for plant growth-promoting (PGP) traits. After several PGP trait screenings; two bacterial consortia that could aid the growth and development of plants were formulated. Further, these screening results were validated by applying selected bacterial strains and the prepared consortia on tomato plants (*Solanum lycopersium*). The growth parameters were evaluated at the plant's germinative, vegetative, and reproductive stages. The biochemical characteristics under the experimental conditions were also examined at various intervals. The results revealed that both our consortia were able to stimulate the growth and development of the host plant. Hence, the formulated bacterial combinations have real potential to become ecologically sustainable biofertilizers.

**Keywords** Plant growth promoting bacteria · Biofertilizer · Tomato · Physiology · Biochemical analysis

## Abbreviations

PGPB Plant Growth Promoting Bacteria

BSA Bovine Serum Albumin

NCBI National Center for Biotechnology Information

IAA Indole-3-Acetic Acid

KC-5 Kanyakumari Culture-5

KC-6 Kanyakumari Culture -6

VLC-4 Vellore Lake Culture -4

BFC Biofertilizer Control

HD Halo Diameter

CD Colony Diameter

HCN Hydrogen Cyanide

ACC 1-Aminocyclopropane-1-Carboxylate

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## Introduction

Over the years, the use of mineral fertilizers to improve crop growth and productivity has been the trend among cultivators. However, excessive use of such chemicals will negatively impact soil fertility, the soil microbiome, and eventually the environment (Gomiero et al. 2011). To this end, agricultural practice is moving toward a more sustainable and environmentally friendly approach. The use of biostimulants in improving genomic and physiological characteristics is a promising practice that was used over the last decades (Katsenios et al. 2021). The application of plant growth-promoting bacteria (PGPB)

as a part of mainstream agricultural practice is an example of a bio-stimulant that is becoming a trend.

The bio-stimulation ability of PGPBs is characterized by the production of various substances (for example; IAA, HCN, etc.). These substances participate either in the direct or indirect mechanisms of PGPB action. The bioactivities such as phytohormone production, nutrient solubilization, and nitrogen fixation along with the production of other bioactive compounds that involve in facilitating the resources for plant growth are categorized as direct mechanisms (Glick 2012). Indirect mechanisms are mainly involved in stimulating induced systemic resistance (ISR). The production of antimicrobial compounds such as hydrogen cyanide, phenazines, lipopeptide biosurfactants, and siderophores can all elevate the plant's immune response toward biotic and abiotic stress. In addition, the production of certain hydrolytic enzymes like protease, chitinases, and glucanases to degrade fungal cell walls or form biofilms on root surfaces that may protect the plant root against soil-borne infections (Vaikuntapu et al. 2014; García-Fraile et al. 2015).

Tomato (*Solanum lycopersicum*) is one of the most cultivated as well as utilized crops worldwide (Bihon et al. 2022). It is widely cultivated across the tropics and sub-tropic regions around the world (Sadashiva et al. 2017). Currently, global warming along with other associated geochemical problems is resulting in the reduction of more than 50% average yields of major crops including tomatoes (Singh et al. 2018). Therefore, sustainable agricultural practices such as the PGPB application possess greater importance in the current scenario of food security.

In the present study, the biochemical ability of several bacterial strains isolated from nutrient-rich environment samples in improving plant growth and development has been elucidated. These results were further validated by conducting seed germination and a pot culture study with *Solanum lycopersicum*. Multiple bacterial isolates were found to be showcasing various PGP traits such as the production of phytohormones, biocontrol activity, and nutrient mineralizing ability. In addition, the compatibility of the selected combinations of bacterial isolates was evaluated. The compatible isolates were used to prepare bacterial consortia and were subjected to seed germination and pot culture experiment with *Solanum lycopersicum*. Furthermore, the physiological and biochemical changes in plants treated with each bacterial formulation were monitored.

## Materials and method

### Bacterial source sample collection

Soil samples were collected from Kanyakumari Seashore (8°07'30.4"N 77°33'57.1" E) and a lake bank in

Vellore- Tamil Nadu (12°58'07.6"N 79°09'32.7" E). These two source samples were selected expecting nutrient solubilizing ability in the native microflora. Samples were collected in plastic bags and brought to Vellore Institute of Technology (VIT), Vellore under cold conditions for isolation of bacteria. Samples were stored at 4 °C until bacteria were isolated for further studies (Gaete et al. 2020).

### Isolation and selection of bacteria

Each soil sample was weighed for 1 g and was serially diluted in 0.85% NaCl (saline). 100 µL of each 10<sup>-3</sup>, 10<sup>-4</sup>, and 10<sup>-5</sup> dilutions were used to spread the plate on Luria–Bertani (LB) agar plates. The plates were incubated at 37 °C. Isolates with dissimilar morphology were picked from the plates and were used to prepare pure culture (Vaikuntapu et al. 2014).

### Screening for plant growth-promoting traits

#### Indole-3- acetic acid (IAA) production: quantitative microplate assay

A total of 15 distinctive pure isolates were subjected to quantifying IAA production as described by Sarwar and Kremer (Sarwar and Kremer 1995). Bacterial isolates were incubated at 28 °C in LB broth containing 0.1% tryptophan. After 48 h the broth was centrifuged at 6000 × g for 10 min. To 150 µL supernatant, 100 µL Salkowski reagent (57 mL of 60% of perchloric acid, 41 mL distilled water, and 2 mL of 0.5 M ferric chloride) were added in a 96-well microplate and was allowed to react for 30 min. The color intensity was measured at 530 nm (Sarker and Al-Rashid 2013).

#### Siderophore production

The siderophore production ability of 15 individual strains was determined using a method described by Schwyn and Neilands (Schwyn and Neilands 1987) with slight modifications. Chrome Azurol S reagent was prepared by mixing 100 mL CAS (121 mg in 100 mL), 20 mL 1 mM FeCl<sub>3</sub> (prepared using 10 mM HCl), and 80 mL hexadecyl trimethyl ammonium bromide (HDTMA) solution (729 mg HDTMA in 400 mL distilled water). The LB agar media was autoclaved and mixed with CAS reagent (10 mL per 100 mL LB agar) and poured into the plates. 4 wells were created in each plate. 20 µL of overnight cultures were injected into the well. The formation of a yellow-colored halo zone around the inoculated wells indicates the bacterial production of siderophore.

### ACC Deaminase production: quantitative microplate assay

1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), an enzyme that actively participates in lowering ethylene levels in the plant is quantified according to Li et al. (Li et al. 2011) protocol. The 15 overnight bacterial cultures were inoculated to Dworkin and Foster (DF) minimal salt medium with  $0.05 \text{ ML}^{-1}$  ACC (aminocyclopropane-1-carboxylic acid) and without ACC as control. The broth cultures were centrifuged at  $8000 \times g$  for 5 min.  $100 \mu\text{L}$  supernatant was diluted with  $900 \mu\text{L}$  DF medium; from which  $60 \mu\text{L}$  was mixed with  $120 \mu\text{L}$  ninhydrin reagent (500 mg ninhydrin, 15 mg ascorbic acid in 60 mL of ethylene glycol, 60 mL of citrate buffer pH 6) in a PCR tube and mixed by pipetting. These PCR tubes were then heated at  $100^\circ\text{C}$  in the water bath for 30 min. After allowing the samples to cool, it was transferred to a 96-well microplate. The optical density was measured at 530 nm.

### Ammonia production

Bacterial strains were inoculated in 3 mL of peptone incubated overnight at  $37^\circ\text{C}$  for 24 h. After incubation  $600 \mu\text{L}$  Nessler's reagent was added to each test tube. The production of yellow to dark brown color was considered to be positive for ammonia production. The bacterial ammonia production was then quantified using a spectrophotometer at 450 nm. Different concentrations of ammonium sulfate ranging from  $100 \mu\text{M}$  to  $500 \mu\text{M}$  were used to prepare the standard curve (Abdelwahed et al. 2022).

### Phosphate solubilization

All 15 bacterial isolates were subjected to a phosphate solubilization assay.  $10 \mu\text{L}$  of each bacterial culture was spot inoculated in National Botanical Research Institutes' -bromophenol blue (NBRIP-BPB) growth medium plate (D-glucose, 10 g;  $\text{Ca}_3(\text{PO}_4)_2$ , 5 g;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 5 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 g; KCl, 0.2 g;  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 g; bromophenol blue, 0.025 g; agar, 15 g; and distilled water, 1L, pH-7.0) and incubated at  $37^\circ\text{C}$  for 7 days. The formation of a clear halo zone around each colony was closely monitored. The halo diameter (HD) and colony diameter (CD) of each colony was measured and the ratio of HD/CD was calculated. A bacterial culture with an HD/CD ratio greater than or equal to 1.5 is considered to have a strong ability to solubilize phosphate, whereas those cultures having a value between 1.0 to 1.5 are considered to be weak phosphate solubilizers (Li et al. 2019).

### Zinc solubilization

The selected bacterial isolates were screened for their ability to solubilize the insoluble zinc compound ZnO. Overnight cultures were spot inoculated in tris minimal media (per liter: Tris-HCl 6.06 g; NaCl 4.68 g; KCl 1.49 g;  $\text{NH}_4\text{Cl}$  1.07 g;  $\text{Na}_2\text{SO}_4$  0.43 g;  $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$  0.2 g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 30 mg, pH 7.0; added 0.1% ZnO (w/v) and 1.5% agar. After inoculation, the plates were incubated in dark conditions at  $30^\circ\text{C}$  for 14 days (Sharma et al. 2011; Kamran et al. 2017). The formation of a clear halo zone indicates zinc solubilization.

### Potassium solubilization

The ability of the strains to solubilize potassium was analyzed by inoculating them in the Aleksandrov agar medium (Himedia). Spot inoculation of  $10 \mu\text{L}$  of each bacterial culture into Aleksandrov agar was done and incubated at  $30^\circ\text{C}$  for 3–4 days (RAJAWAT et al. 2016). The formation of the halo zone indicated potassium solubilization.

### Hydrogen Cyanide (HCN) production

All the 15 isolates were screened for hydrogen cyanide production following the method described by Lorck (1948). Each isolate was streaked on nutrient agar medium supplemented with glycine (4.4 g/L). The lid of the agar plate was covered with a Whatman number 1 filter paper (Previously soaked in a 0.5% picric acid and 2% sodium carbonate w/v solution). Plates were sealed with parafilm and incubated at room temperature for 4 days. The appearance of orange or red coloration in the paper indicates the production of hydrogen cyanide.

### Protease production

Skim milk agar plates prepared by adding 1% sterile skim milk powder to autoclaved nutrient agar were used to detect the bacterial production of proteases. The spot-inoculated plates were incubated overnight at room temperature. The formation of the halo zone around the spot inoculation indicated the bacterial ability to produce protease (Yasmin et al. 2020).

### Compatibility test and consortia preparation

The isolates KC-5, KC-6, VLC-4, and VLC-6 showed superior qualities in PGP screening. Therefore, these isolates were selected for further consortia preparation and plant study. These isolates were streaked on nutrient agar plates in such a way that every culture will cross each other. The plates were incubated at  $37^\circ\text{C}$  for 48 h and the zone of

inhibition around each bacterial colony meeting point was observed and recorded (Prasad and Babu 2017).

### Biochemical and molecular identification of the bacterial isolates

The DNA of each selected strain was isolated using G. HCl (Guanidine Hydrochloride) method (Gupta et al. 2013). Later, 16 s rRNA gene was amplified using a universal primer sequence set (27F and 1492R). The polymerase chain (PCR) reaction was carried out under the condition of initial denaturation 95° C for 5 min, 35 cycles of 95° C for 30 s, 50° C for 30 s, 72° C for 30 s and final extension of 72° C for 10 min. The PCR product was subjected to Sanger sequencing, and obtained sequences were blasted for retrieving similar sequences (Senthilraj et al. 2016). These similar sequences were used for constructing a phylogenetic tree and further identification of the isolates (Ayyaz et al. 2016).

Biochemical tests such as indole, Methyl Red (MR), Voges Proskauer (VP), citrate, catalase, and oxidase tests along with Gram's staining were performed to conform to the lineage of the selected isolates (Chauhan and Jindal 2020).

### Biofilm formation

The ability of each isolate to form biofilm was tested following the method described by O'Toole and Kolter (O'Toole and Kolter 1998) with slight modifications. The 1% of overnight bacterial cultures were grown in a 24-well microtiter plate containing LB broth media. After 72 h the broth was removed and the adhered bacterial cells were washed with sterilized LB media. The washed bacterial cells were stained using 1% crystal violet and incubated for 15 min. The dye was discarded and the cells were rinsed with distilled water. The dye absorbed by the biofilm formed was then dissolved with 70% ethanol and subsequently quantified with a UV spectrophotometer at 590 nm (Yousef-Coronado et al. 2008; Santiago et al. 2017).

### Antibiotic resistance

The selected bacterial isolates were tested for antibiotic resistance against antibiotics namely Rifampicin (10 mcg), Ampicillin (10 mcg), Erythromycin (15 mcg), Kanamycin (30 mcg), Tetracycline (30 mcg), and Chloramphenicol (30 mcg) by the antibiotic sensitivity assay. The bacterial cultures were swabbed on Mueller Hinton agar (MHA) media plates. The antibiotic discs (Himedia, India) were placed above the media surface. The plates were incubated at 37 °C for 24 h. A zone of bacterial growth inhibition around the disc implies the sensitivity of the bacteria toward the

respective antibiotic. The diameter of the zone was measured subsequently (Prakash Singh and Nath Jha 2015).

### Seed germination study

The seed germination study was carried out following the procedure described by Vasseur-Coronado et al. (Vasseur-Coronado et al. 2021) with slight modifications. The isolates namely, KC-5, KC-6, and VLC-4 were used for this study as individual and as consortia along with commercially available *Pseudomonas* sp. based biofertilizer control- bought from farmer's biofertilizers (<https://www.farmersbiofertilizers.com/>). The bacterial cultures for the seed treatment were prepared by inoculating the individual cultures and the consortia in nutrient broth. Overnight cultures were pelleted out by centrifugation. These pellets were dissolved in PBS in such a way that it contains 0.5 OD cells @ 630 nm. Subsequently, the PKM-1 tomato (*Solanum lycopersicum*) variety seeds were disinfected by submerging them in the following solutions: 70% ethanol for 2 min, and 1% sodium hypochlorite (NaOCl) (SDfine, India) for 5 min. These seeds were then subjected to washing with sterile distilled water multiple times. The disinfected seeds were then soaked in either the respective bacterial formulations or in PBS (control group) for 30 min under shaking. The treated seeds are then placed in germination paper moistened with sterile distilled water (25 times its weight). A total of 12 seeds were placed in each germination paper in triplicates for each treatment group. Petri dishes were incubated at 25–30 °C. After 12 days, the physiological parameters were measured and the vigor index was calculated. The seed vigor index was calculated by applying the following formula.

$$\text{Vigour index} = \text{Average seedling length} * \text{Germination rate (\%)}$$

### Pot culture study- physiology and biochemical analysis

The bacterial cultures were prepared by inoculating the individual cultures and the consortia in nutrient broth. After 72 h at 30° C, the obtained cells were pelleted out by centrifuging. These pellets were then washed 3 times in sterile 0.85% NaCl and the concentration of the cells was appropriated to 0.6 OD at 600 nm using the same saline solution. The prepared bacterial suspensions were used to treat the tomato seedlings (Helal et al. 2022).

PKM-1 tomato (*Solanum lycopersicum*) variety seeds were sown and allowed to grow in the seedling tray for one month. The one-month-old seedlings were transplanted into polythene grow bags. Before transplanting, the roots of the seedlings were washed with water to remove the soil around them. These seedlings were then treated with

bacterial cultures (control-no treatment, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and commercially available *Pseudomonas* sp. based biofertilizer control- bought from farmer's biofertilizers (<https://www.farmersbiofertilizers.com/>) by soaking the roots in the respective bacterial suspension. After transplantation, the plant grow bags was kept in a completely randomized design with 5 replications in each treatment including control. The transplanted plants were again treated with the respective bacterial culture treatments after 15 days of treatment.

### Plant-Physiological analysis

Physiological parameters like the shoot length, number of branches, and the number of leaves, per plant, were measured in different intervals after transplantation until the stage of reproduction.

### Plant-Biochemical analysis

Biochemical analyses such as the quantification of carbohydrates, protein, phenol, and pigments were done on the 30th, 45th, and 60th day after transplantation.

### Estimation of carbohydrates

Fresh tomato leaves weighing 40 mg were picked from each treatment group, washed with distilled water, and squashed in a mortar and pestle with 2 mL distilled water. The mixture was heated at 70° C for 20 min followed by centrifugation at 5000 × g at 4° C for 15 min (Karthik et al. 2020). The resulting supernatant was then used for the quantification of carbohydrates by using a phenol–sulphuric acid assay (Chow and Landhäusser 2004) in a 96-well microtiter plate.

To 100 µL of each leaf extract 100 µL of 5% phenol was added along with 500 µL of concentrated sulphuric acid in ice-cold conditions. The mixture was then brought to room temperature by heating it in a hot water bath for 20 min. The samples were then allowed to cool and the absorbance was measured @ 490 nm. Glucose standard concentrations ranging from 0.025 to 2 mg/mL were used to plot the absorbance against the concentration standard curve. Using the standard graph, the unknown carbohydrate concentration in plant samples was estimated. The experiment was done in replications and the mean value was considered.

### Estimation of protein

Fresh leaf samples weighing 40 mg were ground with 3 mL water in a mortar and pestle. The mixture was centrifuged at 14000 × g at 4° C for 10 min (Mathur et al. 2015). The resulting supernatant was used for the estimation of protein using the BCA protein quantification assay kit (TAKARA, INDIA)

following manufacturer protocol. Bovine Serum Albumin (BSA) concentrations ranging from 0.025 to 2 mg/mL were used to plot absorbance against the concentration standard curve. The experiment was done in triplicates and the mean value was considered (Sun et al. 2007).

### Estimation of phenol

Fresh leaf samples weighing 40 mg were crushed with 2 mL of 80% ethanol followed by the centrifugation of the mixture at 14,000 × g at 4° C for 20 min. The supernatant was separated and used as the leaf extract for the estimation of phenol. Phenol in the sample was quantified using Folin Ciocalteu (FC) reagent (Maheswari and Elakkiya 2014). In a 1.5 ml tube, 100 µL of each sample and 200 µL of FC reagent were added and mixed. The mixture was then neutralized with 400 µL 7.5% sodium carbonate and was incubated at room temperature for 30 min with shaking for color development. 200 µL of the reaction mixture was transferred into a 96-well plate. The absorbance of the resulting blue color was measured at 725 nm. Gallic acid concentrations ranging from 0.025 to 2 mg/mL were used to plot absorbance against the concentration standard curve. The experiment was done in triplicates and the mean value was considered.

### Estimation of pigments

Leaf samples weighing 100 mg were ground with 10 mL of 80% acetone in a mortar and pestle. The mixture was then centrifuged at 14,000 × g at 4° C for 10 min. The optical density of the supernatant was analyzed at 645 nm, 663 nm, and 470 nm. Based on the OD values obtained the concentrations of chlorophyll a (Chl A), chlorophyll b (Chl B), and carotenoids were calculated (Sarkar et al. 2018). The experiment was done in triplicates and the mean value was considered.

## Results

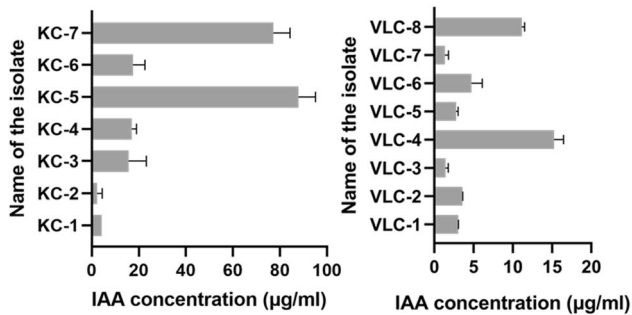
### Isolation and selection of bacterial cultures

Each sample source was selected for scientific reasons. A total of 15 morphologically distinctive bacterial isolates were selected from both sample sources for the screening of PGP properties.

### Screening for plant growth-promoting traits

#### Indole-3- acetic acid (IAA) production: quantitative microplate assay

The ability of the selected 15 isolates in producing IAA, siderophore, and ACC deaminase was investigated

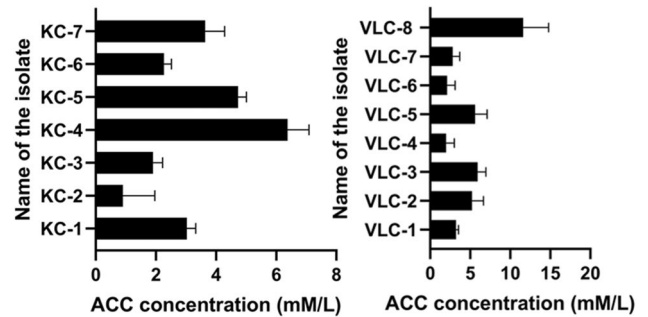


**Fig. 1** Concentration of Indole-3- acetic acid (IAA) produced by each strain

predominately. Out of the 15 strains; KC-5 ( $87.93 \mu\text{g mL}^{-1}$ ), and KC-7 ( $77.18 \mu\text{g mL}^{-1}$ ) showed the best performance in IAA production. However, KC-3 ( $15.67 \mu\text{g mL}^{-1}$ ), VLC-4 ( $15.26 \mu\text{g mL}^{-1}$ ), and VLC-8 ( $11.17 \mu\text{g mL}^{-1}$ ) were found to be moderate IAA producers (Fig. 1).

### Siderophore production

The CAS-qualitative agar test for siderophore production revealed a yellow zone around siderophore-producing bacterial strains (Fig. 2). Isolates namely- KC-2 and KC-5 showed the formation of a strong halo after 24 h of incubation. Isolates like KC-1, KC-4, KC-6, VLC-1, VLC-4, and VLC-6 showed gradual zone formation from mild to strong in the 48 h period. The rest of the bacterial isolates showed no or

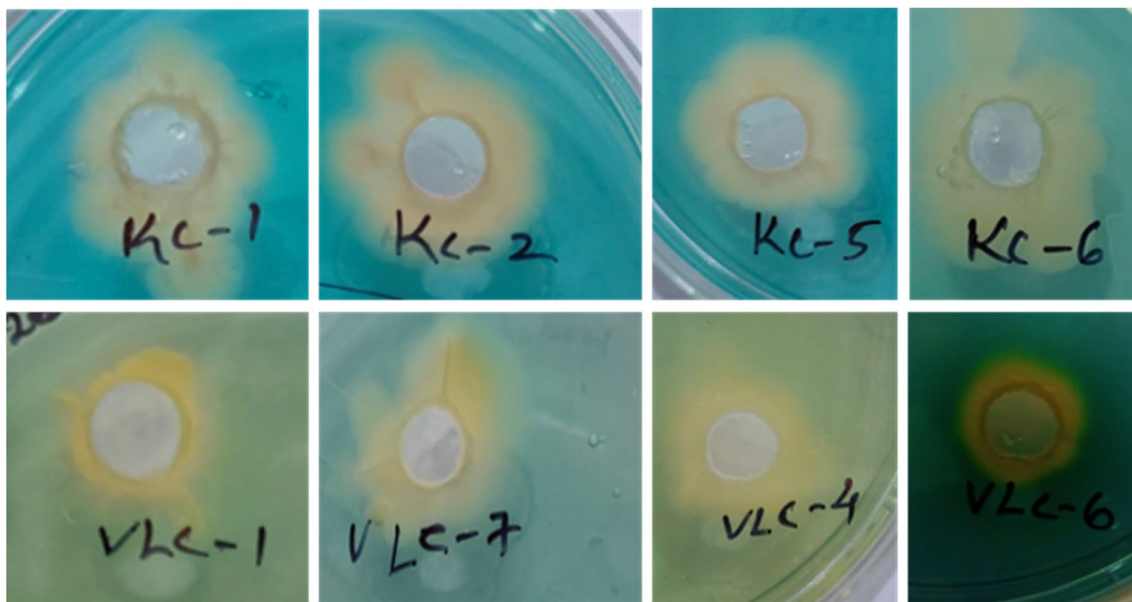


**Fig. 3** Concentration of ACC deaminase produced by each strain

negligible zone formation implying the inability of these strains to produce siderophore.

### ACC Deaminase production: quantitative microplate assay

The PCR-plate ninhydrin ACC assay determined the ability of the selected 15 cultures in producing ACC deaminase. All 15 isolates were found to be having some ACC deaminase activity as the average final ACC concentration in the broth after 24 h incubation was around  $5 \text{mM L}^{-1}$  (Fig. 3). However, certain isolates like VLC-4, VLC-6, KC-2, KC-3, KC-5, and KC-6 showed better ACC utilization (final ACC concentration  $1.5 \text{mM L}^{-1}$  or below), implying better performance for ACC deaminase production.



**Fig. 2** Formation of yellow zones indicating production of siderophore

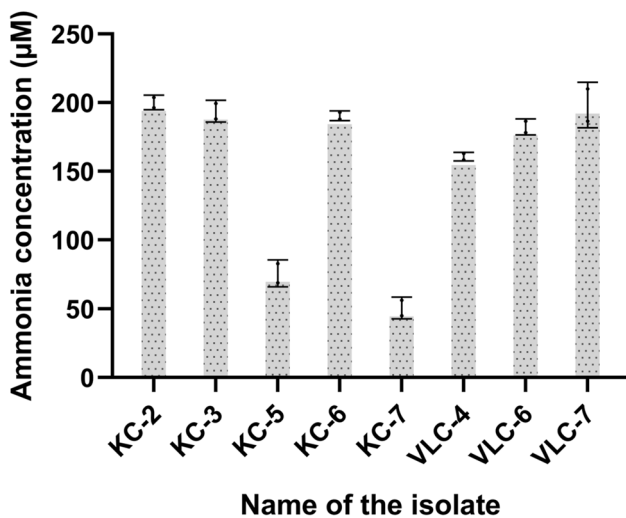


Fig. 4 Concentration of Ammonia produced by each strain

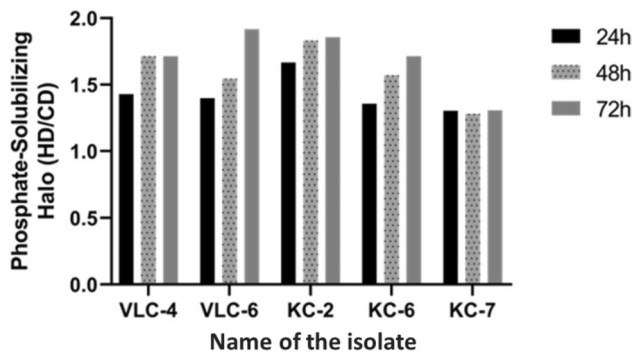


Fig. 5 Growth rate of phosphate solubilizing halo (HD/CD)

### Ammonia production

The qualitative test showed that 8 isolates tested were positive for ammonia production (Fig. 4). All isolates except KC-5 and KC-7 showed an average ammonia production of 150 µM.

### Phosphate solubilization

Among the 15 isolates that were subjected to phosphate solubilization assay, 5 of the isolates showed superiority in mineralizing phosphate (HD/CD above 1.5). These 5 isolates (namely KC-7, VLC-4, VLC-6, KC-2, and KC-6) were then closely monitored for their solubilizing efficiency (Fig. 5). The isolates CSC-1 and KC-6 showed a steady growth rate of phosphate solubilizing halo (HD/CD) over the 72 h period. VLC-6 showed a third-day spike in the halo, implying the gradual attaining of the mineralization

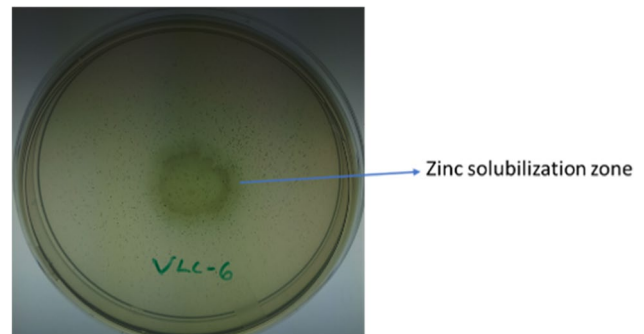


Fig. 6 Formation of zinc solubilization halo by VLC-6 strain

target. KC-7 and KC-2 showed no specific growth rate of halo over time, even though KC-2 showed a better HD/CD ratio. VLC-4 seemed to end its phosphate mineralization on the second day since there was not much change observed in the halo.

### Zinc solubilization

Here we screened the selected cultures for their ability to solubilize ZnO. Out of the 15 isolates screened for zinc solubilization in ZnO-incorporated tris minimal media, only VLC-6 was found to show positive zone formation (Fig. 6).

### Potassium solubilization

The zone formed around the cultures suggested that 4 (namely, KC-7, VLC-4, VLC-6, and KC-6) out of the 15 isolates were found to have the ability to solubilize the insoluble form of potassium (Potassium aluminum silicate) in the Aleksandrov medium (Fig. 7).

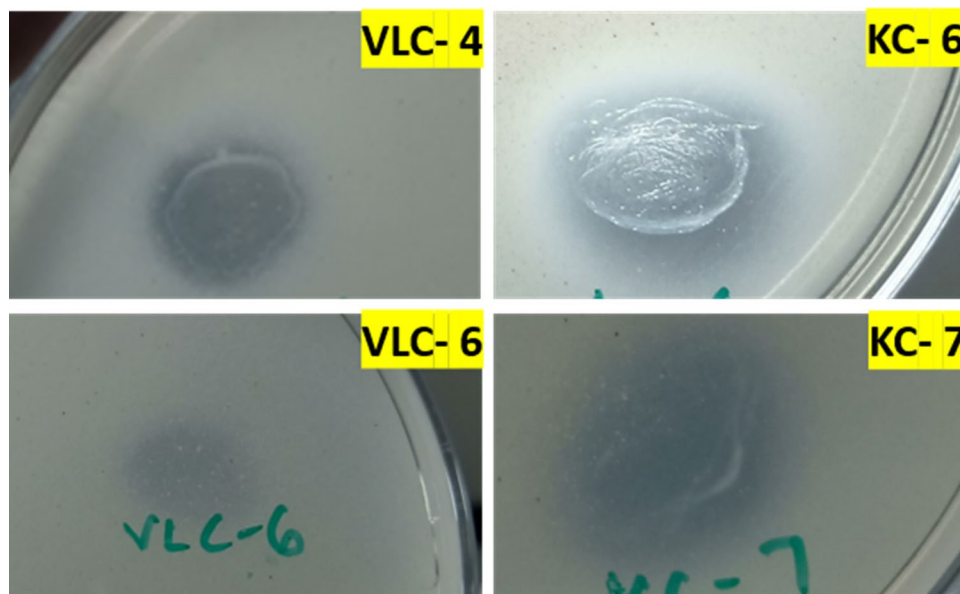
### HCN production

The picric acid dye present in the filter paper undergoes molecular rearrangement when it reacts with the HCN produced by the bacteria- changing its coloration from yellow to orange. Among the 15 isolates screened only VLC-6 was found to be able to produce HCN (Fig. 8).

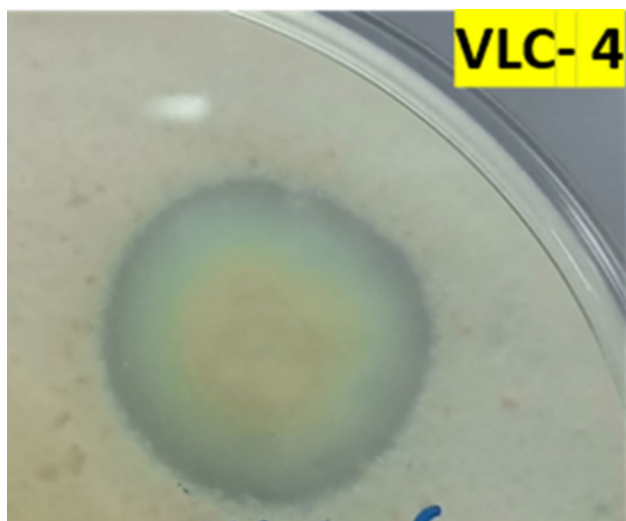
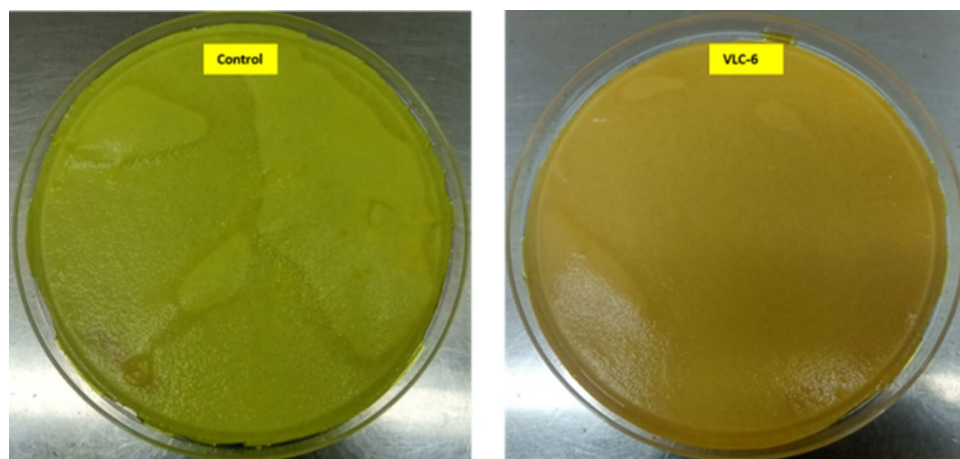
### Protease production

Out of the 15 isolates screened for protease production, VLC-4 was found to be the only isolate that produces protease (Fig. 9).

**Fig. 7** Formation of potassium solubilization halo by bacterial strain



**Fig. 8** Formation of orange coloration indicating production of Hydrogen Cyanide (HCN) by VLC-6 strain



**Fig. 9** Hydrolysis of skim milk from the media indicating production of protease by VLC-4 strain

### Identification of the bacterial isolates

The constructed phylogenetic trees revealed that the bacterial isolate VLC-4, KC-5, KC-6 and VLC-6 were closer to *Enterobacter* sp., *Lysinibacillus* sp., *Acinetobacter* sp. and *Pseudomonas aeruginosa* respectively (Fig. S1). The sequences of the respective isolate were then submitted to the NCBI database and accession numbers were retrieved as follows; *Enterobacter* Sp: OP050456, *Lysinibacillus* sp.: OP070953, *Acinetobacter* sp.: OP080714 and *Pseudomonas aeruginosa*: OP055908. The biochemical Characterization of the isolates (Table 1) further validated the molecular identification.

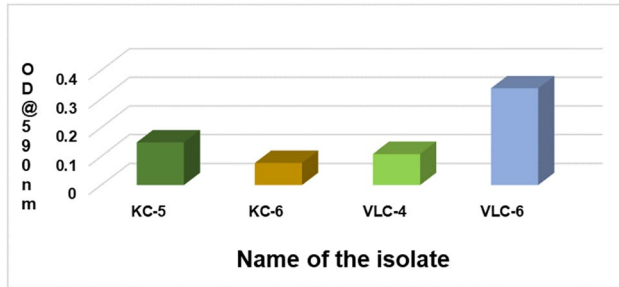
### Biofilm formation

Among the isolates KC-5, KC-6, VLC-4, and VLC-6; VLC-6 and KC-5 were seen to be having a greater ability



**Table 1** Biochemical test results of the selected bacterial isolates. The presented results support and validates the molecular identification

Isolate	Gram’s test	Indole	MR	VP	Citrate	Catalase	Oxidase
KC-5	+	-	-	-	+	+	+
KC-6	-	-	+	-	+	+	-
VLC-4	-	-	-	+	+	+	-
VLC-6	-	-	-	-	+	+	+



**Fig. 10** Biofilm formation by bacterial isolates

to form a biofilm with 0.4 and 0.15 OD respectively. However, KC-6 and VLC-4 are moderate biofilm producers (Fig. 10).

**Antibiotic resistance**

VLC-6 was seen to be resistant to most of the antibiotics which were tested. VLC-4 and KC-6 were showing moderate susceptibility to most of the antibiotics. KC-5 was susceptible to all the antibiotics tested (Table 2).

**Compatibility test and consortia preparation**

KC-5, KC-6, VLC-4, and VLC-6 were subjected to compatibility tests in different combinations, among which VLC-6 was seen inhibiting the growths of KC-5 & KC-6. However, KC-5 + KC-6, KC-5 + VLC-4, and VLC-4 + VLC-6 were compatible with each other (Fig. 11). KC-5 + KC-6 and KC-5 + VLC-4 were selected for further plant study as VLC-6 was identified to be *Pseudomonas aeruginosa* which is reported to be a human pathogen.

**Table 2** Antibiotic resistance test results of the selected bacterial isolates suggesting adaptability of these isolates for potential agricultural applications

Name of the isolate	Rifampicin 5 (mm)	Kanamycin 30 (mm)	Chloramphenicol 30 (mm)	Tetracycline 30 (mm)	Erythromycin 15 (mm)	Ampicillin 10 (mm)
KC-5	25	30	35	40	40	40
KC-6	10	20	15	20	20	-
VLC-4	-	18	20	15	12	12
VLC-6	-	10	-	7	8	-

**Seed germination study**

The measurement of shoot length, root length, and seedling length was noted down (Table 3). Subsequently the vigor index was also calculated to characterize the impact of the bacterial treatments on the germination of tomato seeds. The results showcased that all the bacterial treatments considerably promoted ‘germination’ when compared with the control group (Fig. S2). The highest vigor index was found to be shown by the KC-6 (983.98) and biofertilizer control group (957.27). However, the formulated consortia KC-5 + KC-6 and KC-5 + VLC-4 showed average performance with a vigor index of 785.80 and 700.31, respectively.

**Pot culture study- physiology and biochemical analysis**

**Physiological analysis**

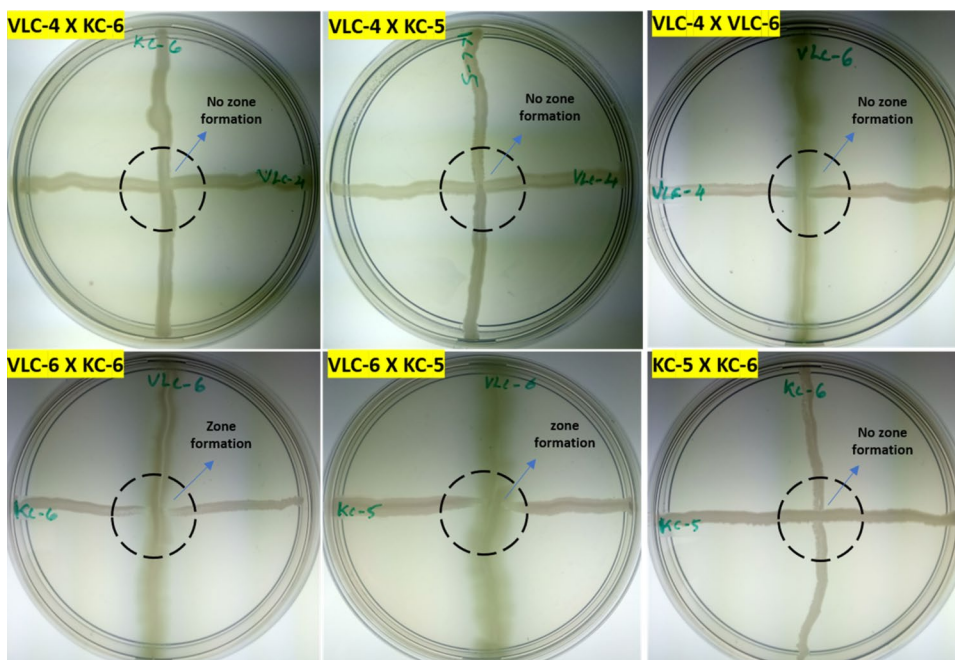
The measurement of plant height, number of branches, and number of leaves on the 10th, 20th, 30th, and 50th days were observed to understand the influence of bacterial treatments on plant growth (Table 4). The results suggested that the application of the consortium KC-5 + KC-6 and the biofertilizer control have the highest values with statistically significant ( $p < 0.05$ ) differences compared to all other treatment groups (Fig. S3). Moreover, all PGPB treatments had statistically significant differences compared to that of the control.

**Biochemical analysis**

**Carbohydrates**

The concentrations of carbohydrates were better in all the treatment groups when compared to the control group at each

**Fig. 11** cultural compatibility test; the formation a zone in the meeting junction between the two isolate shows the inhibition of one bacterial growth by the other. Here, VLC-6 is seen inhibiting the growth of KC-5



**Table 3** Seed germination test results under various bacterial treatments; both individually and as consortia

Parameter	Control	KC-5	KC-6	VLC-4	KC-5 + KC-6	KC-5 + VLC-4	Biofertilizer control
Shoot length	2.95 ± 1.24	4.82 ± 0.98a	5.01 ± 0.33a	4.77 ± 0.54a	4.98 ± 0.82a	5.08 ± 0.58a	3.82 ± 1.08cef
Root length	1.98 ± 1.59	3.73 ± 1.93	6.04 ± 2.06a	3.83 ± 1.64	3.43 ± 1.50	4.38 ± 2.40	8.67 ± 3.16abdefg
Seedling length	4.87 ± 2.58	8.59 ± 2.47a	11.25 ± 2.23a	8.89 ± 1.97a	8.30 ± 2.04a	8.38 ± 1.84a	12.76 ± 3.96abdefg
Vigour Index	365.45	778.55	983.98	722.39	700.31	785.80	957.27

Data in the table represents mean ± SD and the variable (a,b,c,d,e,f) indicates statistically significant ( $p < 0.05$ ). “a” implies control vs KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “b” implies KC-5 vs KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “c” implies KC-6 vs VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “d” implies VLC-4 vs KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “e” implies KC-5 + KC-6 vs KC-5 + VLC-4, and Biofertilizer control; “f” KC-5 + VLC-4 vs Biofertilizer control

of the time intervals the experiment was conducted (Fig. 12). On the 30th day, to a 0.264 mg/mL of control; 0.544, 0.563, 0.301, 0.574, 0.384, and 0.611 mg/mL carbohydrates were present in KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. On the 45th day, the values changed to a 0.38 mg/mL control; 0.38, 0.485, 0.465, 0.47, 0.59, 0.58, and 0.615 mg/mL in KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. In the case of the 60th day, the values were; to a 0.279 mg/mL control; 0.362, 0.343, 0.376, 0.515, 0.460, and 0.544 mg/mL in KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively.

**Protein**

The protein content in each treatment group was estimated to be similar on the 30th day (Fig. 12). A considerable change

in protein content was observed on days 45 and 60. On the 30th day, the values were 2.043, 1.962, 1.981, 1.908, 2.138, 2.119, and 2.172 mg/mL in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. On the 45th day, the values were 0.4471, 0.930, 1.456, 0.585, 0.807, 1.031, and 0.982 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. In the case of 60th day, the protein was estimated to be 1.742, 1.952, 1.835, 1.843, 1.955, 1.897, and 1.773 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively.

**Phenol**

The phenol content steadily increased with each culture treatment when compared to the control group on each of the 3-time intervals the experiment was performed (Fig. 12). On the 30th day, to a 0.105 mg/mL of

**Table 4** Physiological analysis of plants under various bacterial treatments; both individually and as consortia

Parameter	Phase	Control	KC-5	KC-6	VLC-4	KC-5 + KC-6	KC-5 + VLC-4	Biofertilizer control
Plant height	10th day	2±0	2±0	2±0	3±0.5	3.17±0.29 abc	2.83±0.58	3±0.5
	20th day	8.17±1.26	11.83±0.76	8.83±1.04	8.67±0.58c	14.33±1.15acd	14.00±2.00acde	12.67±.58acdef
	30th day	17.83±1.04	28.17±0.76	20.67±2.08b	18.17±0.76b	25.67±1.15acd	22.17±1.61abd	27.33±1.53acdf
	50th day	40.67±2.08a	50.67±3.06	44.00±2.65	43.50±1.50bc	51.33±2.08acd	53.07±2.64acde	52.67±2.52acdef
Number of branches	10th day	4.00±1.00	3.33±0.58	3.33±0.58	3.33±0.58	4.33±0.58	4.00±1.00	4.67±1.15
	20th day	5.00±1.00	6.33±1.53	6.00±0	4.00±0	6.67±0.58	7.00±1.00	7.67±2.08d
	30th day	8.67±1.15	11.00±2.00	9.33±1.15	7.67±1.15	14.00±1.73d	11.00±4.58	16.00±2.00acd
	50th day	11.67±2.08	13.67±0.58	12.00±3.00	11.33±3.51	20.33±1.53abcd	17.33±0.58d	18.67±1.53acd
Number of leaves	10th day	9.33±2.08	10.33±0.58	12.00±2.00	16.33±2.52	16.33±2.52	13.67±4.04	17.33±4.04a
	20th day	20.33±5.51	26.00±1.00	27.67±2.08	16.00±1.73bc	34.67±2.52abd	36.00±3.00abd	30.67±3.21ad
	30th day	48.33±5.03a	82.67±9.71	62.67±6.81	50.67±3.79b	102.33±13.05acd	91.67±6.66acd	100.00±6.00acd
	50th day	99.67±14.05	136.67±12.01	97.00±17.52	81.67±15.37b	134.67±11.02d	113.33±23.80	121.67±16.07
Average number of fruits per plant	-	6.6±0.84	9.6±1.1	7.8±0.89	7.2±0.55	9.6±1.79	9±1	8.4±1.3
Total Yield in gs (5 plants)	-	718.86±17.2	1440.75±27.14	1045.02±26.81	1086.57±27.79	1387.11±57.55	1475.33±29.19	1179.60±24.81
Average weight of the fruit	-	25.02±8.7	31.27±8.47	30.89±6.34	33.68±8.81	32.45±6.80	33.38±4.94	29.50±4.69

Data in the table represents mean ±SD and the variable (a,b,c,d,e,f) indicates statistically significant (p<0.05). “a” implies control vs KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “b” implies KC-5 vs KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “c” implies KC-6 vs VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “d” implies VLC-4 vs KC-5 + KC-6, KC-5 + VLC-4, and Biofertilizer control; “e” implies KC-5 + KC-6 vs KC-5 + VLC-4, and Biofertilizer control; “f” KC-5 + VLC-4 vs Biofertilizer control

control; 0.145, 0.115, 0.136, 0.125, 0.144, and 0.131 mg/mL carbohydrates were present in KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. On the 45th day, the values changed to a 0.484 mg/mL control; 0.753, 0.832, 0.730, 0.573, 0.572, and 0.869 mg/mL in KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. In the case of the 60th day, the values were; to a 0.091 mg/mL control; 0.258, 0.291, 0.190, 0.379, 0.255, and 0.402 mg/mL in KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively.

**Pigments**

The estimated Chlorophyll a, chlorophyll b, and carotenoid contents in all the treatment groups showed a significant increase influence of the treated isolates in each of the 3-time intervals except on a few occasions (Fig. 13). On the 30th day, the values of chl A were 0.494, 0.762, 0.523, 0.612, 0.502, 0.575, 0.466 mg/mL in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. On the 45th day, the values were 0.348, 0.638, 0.697, 0.527, 0.682, 0.616, and 0.299 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. In the case of the 60th day, the chl A was

estimated to be 0.355, 0.601, 0.563, 0.456, 0.633, 0.479, and 0.533 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively.

On the 30th day, the values of chl B were 0.884, 1.180, 0.918, 0.692, 1.050, 1.01, and 0.887 mg/mL in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. On the 45th day, the values were 0.651, 1.183, 1.184, 0.601, 1.377, 1.197, and 0.930 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. In the case of the 60th day, the chl B was estimated to be 0.668, 1.107, 1.040, 0.861, 1.134, 0.910, and 0.966 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively.

On the 30th day, the values of carotenoids were found to be 0.442, 0.657, 0.432, 0.766, 0.587, 0.498, and 0.386 mg/mL in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. On the 45th day, the values were 0.419, 0.538, 0.579, 0.386, 0.684, 0.522, and 0.438 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively. In the case of the 60th day, the carotenoids were estimated to be 0.415, 0.542, 0.535, 0.455, 0.567, 0.429, and 0.538 in control, KC-5, KC-6, VLC-4, KC-5 + KC-6, KC-5 + VLC-4, and biofertilizer control respectively.

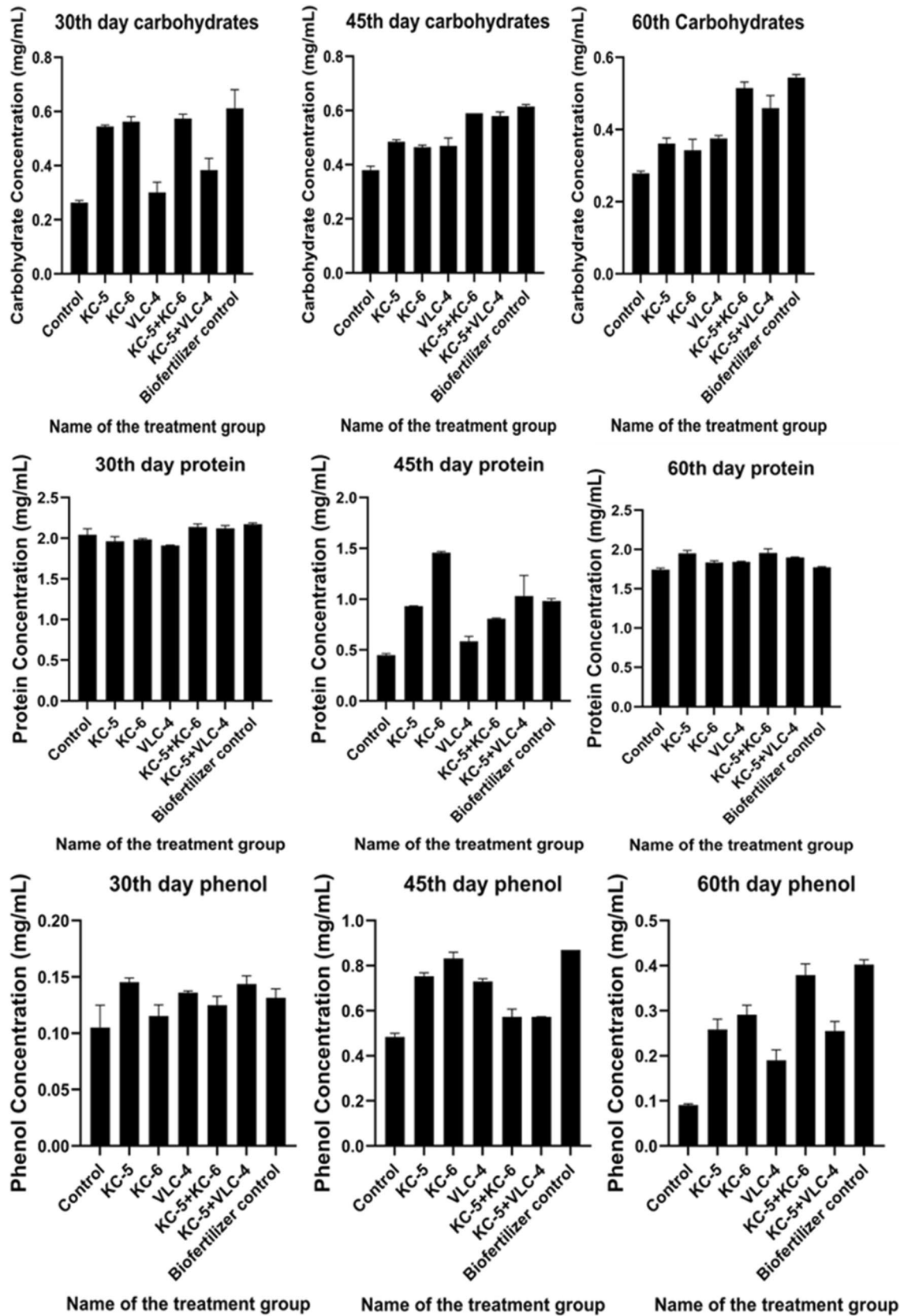
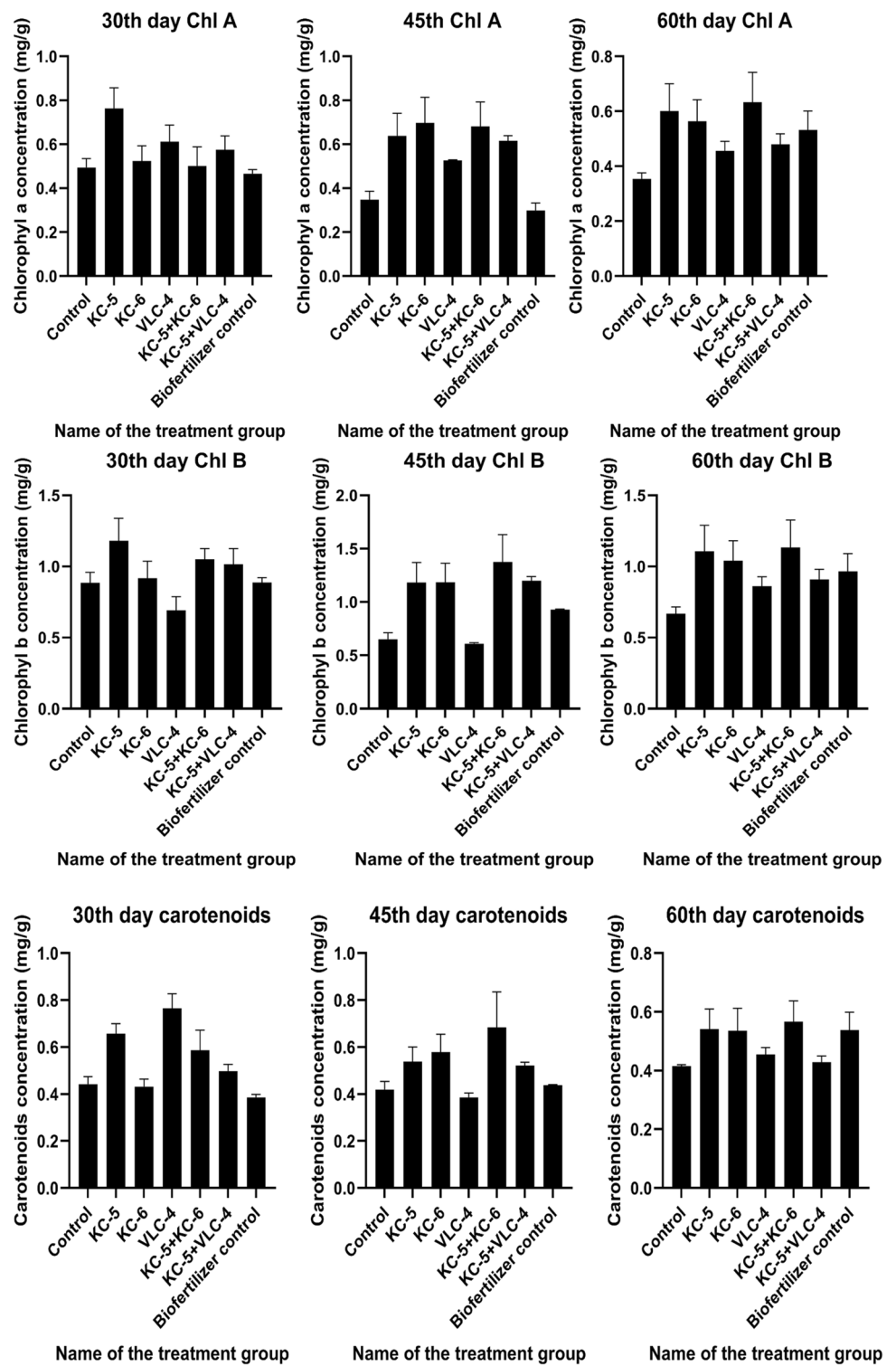


Fig. 12 Biochemical test results of carbohydrate, protein and phenol quantification

**Fig. 13** Biochemical test results of pigments (chlorophyll a, chlorophyll b and carotenoids) quantification



### Discussion

The positive relationship of plants with the soil microbiome is studied extensively through the years to be having a potential future in the sustainability of the agricultural sector (Stamenković et al. 2018). Moreover, any PGPBs related

scientific reports have suggested that tomato is a suitable crop for studying plant-microbial interactions (Ahirwar et al. 2015; Katsenios et al. 2021).

The results obtained in this study also demonstrated that the application of both the formulated PGPB consortia enhanced the growth and development of *Solanum*

*lycopersicum*. In the present study, we isolated bacteria from nutrient-rich soil samples collected from Kanyakumari and Vellore. A total of 15 distinctive bacterial isolates were selected from the spread plates. These isolates were screened for PGPB characteristics such as the production of IAA, Siderophore, ACC deaminase, ammonia, HCN, and protease; solubilization of Phosphate, potassium, and zinc.

IAA (3-indoleacetic acid) is a plant hormone that is commonly known as auxin. Auxin has an influential role in plant growth and development; majorly in vascular differentiation, root architecture, and disease signaling. Plants generally produce this phytohormone in its various parts, while the soil microflora also contributes its share (Duca et al. 2014; Saxena 2014). In this context, the application of our bacterial combinations may have provided an additional supply of IAA which may have supported the growth and development of the host *Solanum lycopersicum*. Similarly, the production of siderophore -an iron-chelating compound that aids the plant in iron absorption, was evaluated. Siderophore is also a known anti-fungal agent and can also help bacteria to colonize the rhizosphere (Saha et al. 2016). 1-aminocyclopropane-1-carboxylic acid (ACC) is a precursor molecule of ethylene (plant stress-responsive hormone that participates in stress-responsive inhibition of plant growth). ACC deaminase enzyme catalyzes the conversion of ACC to ammonia and  $\alpha$ -ketobutyrate. Bacterial ACC deaminase promotes plant growth by reducing plant ACC level and eventually ethylene level thereby ameliorating plant growth inhibition encountered due to ethylene production (Moon and Ali 2022). Ammonia production by bacteria is associated with nitrogen supplementation to the host plants (Bhattacharyya et al. 2020). At times certain nutrients present in the soil may be in an unavailable (insoluble) form to plants. Some genera of bacteria can mineralize this unavailable form of such nutrients to the available form (Granada et al. 2018). Here in this study, we evaluated the ability of our isolates in solubilizing nutrients like phosphate, zinc, and potassium. The production of protease, HCN (Hydrogen Cyanide), and siderophore; and the formation of biofilm are associated with the bacterial aiding of plants for ISR. Protease is an enzyme that hydrolyses proteins and peptides which compose the cell wall of plant pathogens. Likewise, HCN is also a biocontrol agent as it is ascribed to be toxic against plant pathogens (Yasmin et al. 2020). Bacterial biofilm take part in biocontrol of phytopathogens. In addition, they could also enhance nutrient availability to plants and mitigate abiotic stress (Haque et al. 2020). Therefore, the application of bacterial cultures that produce the above-mentioned compounds is expected to benefit the growth and development of plants.

The isolates KC-5, KC-6, VLC-4, and VLC-6 were short-listed by the screening and they were subsequently found to be closely related to NR\_042073 *Lysinibacillus sphaericus* (94.99% similarity), NR\_117931.1 *Acinetobacter nosocomialis* (95.13% similarity), NR\_044978.1 *Enterobacter cloacae* subsp. *dissolvens* (95.66% similarity), and NR\_113599.1 *Pseudomonas aeruginosa* (96.81% similarity) respectively (Fig. S1). Species level identification of KC-5, KC-6 and VLC-4 was not possible, since the blast similarity index was below the threshold (97%) (Aw et al. 2016; Nguyen et al. 2016). However, the similarity index of VLC-6 was nearly the threshold value. Moreover, it showed the biochemical characteristics of *Pseudomonas aeruginosa*. Thus, confirming the molecular identification.

The compatibility test for each of these four bacterial isolates was carried out in different combinations. VLC-6 was found to inhibit the growth of KC-5 and KC-6. Henceforth, KC-5 + KC-6 and KC-5 + VLC-4 combinations were selected for the further plant application study.

The positive impact of our consortia on the germination and vigor of tomato seeds was evaluated by a seed germination study. All bacterial treatment formulations at least doubled the seed vigor index when compared to the control group. However, isolate KC-6 was found to promote seed vigor when treated individually rather than as a consortium. This phenomenon may have happened as in some instances PGPB mixtures do not play synergistic roles in growth promotion (Kang et al. 2014; Lobo et al. 2022).

Plants inoculated with both of our combinations showed better physiological characteristics compared to the control group. However, the application with the consortium KC-5 + KC-6 was found to significantly influence the plant height, number of branches, and number of leaves. This consortium was equally influential in plant physiology like the commercially available biofertilizer control used in this study.

Plant biochemical tests namely, estimation of carbohydrates, protein, phenol, and pigments were performed in 15 days intervals to know the influence of the treated bacterial isolates at different times of growth and development. The concentration of carbohydrates was clearly improved by the application of the formulated consortia. However, KC-5 + KC-6 were found to be stimulating more production of carbohydrates when compared with KC-5 + VLC-4. The 30th-day carbohydrate content suggests that isolate VLC-4 is comparatively weaker improving the carbohydrate content. The applied culture treatments were found not to influence the plants till the 30th day as there was no significant change in any of the treatments including the biofertilizer control when compared to the protein content in control. However, this scenario seems to be changed on 45th and 60th-day protein estimations; as protein content in the control plant was found to be reduced to below 0.5 mg/mL. However, plants applied with bacterial formulations were found to conserve the protein

content. Among the culture treatments, KC-6 when applied alone showed the highest influence on protein production. As a consortium KC-5 + VLC-4 was found to be better than its competitor. A clear influence of the culture treatments on the phenol content level was seen across each time interval quantification. However, the influence rate varied from time to time. In the case of pigments, in both the consortia, the formulation was found to be improving all the pigment contents significantly better than the biofertilizer control.

## Conclusion

In conclusion, the formulated consortia KC-5 + KC-6 and KC-5 + VLC-4 considerably enhanced the growth and development of *Solanum lycopersicum*. When compared to an already marketized bacterial bio fertilizer, our consortia were found to be showing equally good performance in various levels of plant growth promotion. Still, the influence of these consortia on the plants is required to be further explored on a molecular level for a complete understanding of their positive effects on plants for sustainable biofertilizer development.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11756-024-01663-z>.

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**Authors contribution** A T Naveen conceived, designed and performed the research experiments and wrote the manuscript under the direction of R Bharath, R Vidya and R Sudhakaran. Rajan, Kittichon U-taynapun, and Nion Chirapongsatunkul proofread and validated the results and analysis. All authors read and approved the manuscript.

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**Data availability** The genome sequences of KC-5, KC-6, VLC-4 and VLC-6 strains analysed during the current study are available in the GenBank repository.

[<https://www.ncbi.nlm.nih.gov/nucleotide/2277478657>, <https://www.ncbi.nlm.nih.gov/nucleotide/2277723799>, <https://www.ncbi.nlm.nih.gov/nucleotide/2276735244>, <https://www.ncbi.nlm.nih.gov/nucleotide/2277100149>].

## Declarations

**Conflicts of interest** The authors declare that there were no competing interests either financial or non-financial.

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